Mucosal transmission and pathogenesis of chronic wasting disease in ferrets

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Chronic wasting disease (CWD) of cervids is almost certainly transmitted by mucosal contact with the causative prion, whether by direct (animal-to-animal) or indirect (environmental) means. Yet the sites and mechanisms of prion entry remain to be further understood. This study sought to extend this understanding by demonstrating that ferrets exposed to CWD via several mucosal routes developed infection, CWD prion protein (PrP^{CWD}) amplification in lymphoid tissues, neural invasion and florid transmissible spongiform encephalopathy lesions resembling those in native cervid hosts. The ferrets developed extensive PrP^{CWD} accumulation in the nervous system, retina and olfactory epithelium, with lesser deposition in tongue, muscle, salivary gland and the vomeronasal organ. PrP^{CWD} accumulation in mucosal sites, including upper respiratory tract epithelium, olfactory epithelium and intestinal Peyer's patches, make the shedding of prions by infected ferrets plausible. It was also observed that regionally targeted exposure of the nasopharyngeal mucosa resulted in an increased attack rate when compared with oral exposure. The latter finding suggests that nasal exposure enhances permissiveness to CWD infection. The ferret model has further potential for investigation of portals for initiation of CWD infection.

INTRODUCTION

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) affecting mule deer, white-tailed deer, elk and moose (Baeten *et al.*, 2007; Sigurdson, 2008; Williams, 2005; Williams & Miller, 2002). CWD has been detected in both farmed and free-ranging cervid species in 22 states in the USA and two Canadian provinces, and in South Korea (Kim *et al.*, 2005; Sohn *et al.*, 2002). Although first detected in northern Colorado and southern Wyoming, its aetiological origin remains uncertain (Williams, 2005; Williams & Young, 1980). The facile progressive spread of CWD has called attention to its mechanism of transmission and its potential to transgress species barriers. Whilst the capacity of CWD prions to infect several non-cervid species (including sheep, goats, voles, field mice, hamsters, ferrets, cynomolgus macaques and squirrel monkeys) after intracerebral (i.c.) inoculation has been reported (Bartz *et al.*, 1998; Hamir *et al.*, 2005, 2006; Heisey *et al.*, 2010; Kurt *et al.*, 2011; Marsh *et al.*,

2005; Race *et al.*, 2009; Raymond *et al.*, 2007), much less is known regarding the susceptibility of predator/scavenger species such as mustelids, canids and felids to CWD following presumed natural, oral–nasal exposure.

CWD has helped to increase interest in mucosal and haematogenous routes of prion infection (Andréoletti *et al.*, 2000; Haley *et al.*, 2009a; Haybaeck *et al.*, 2011; Kincaid *et al.*, 2012; Maignien *et al.*, 1999; Sigurdson *et al.*, 1999). Studies of CWD susceptibility in sympatric rodent and mustelid species suggest their potential to contribute to CWD epidemiology (Heisey *et al.*, 2010; Kurt *et al.*, 2009). Moreover, several studies in the transmissible mink encephalopathy (TME) and scrapie systems have focused attention on the nasal cavity and associated epithelia (Bessen *et al.*, 2010; Denkers *et al.*, 2010; Kincaid & Bartz, 2007; Kincaid *et al.*, 2012; Sbriccoli *et al.*, 2009) as conduits for prion entry and exit. Thus, the pharyngeal cavity, including its communications with the upper respiratory tract and associated lymphoid structures, presents a variety of possibilities for transduction, excretion and transmission of CWD prions.

A supplementary table is available with the online version of this paper.

Bartz *et al.* (1998) first demonstrated that ferrets are susceptible to CWD following i.c. inoculation. Sigurdson *et al.* (2008) demonstrated a species barrier following oral challenge with deer-origin CWD prions. Subsequent work by Perrott *et al.* (2012) demonstrated that this barrier was obviated upon second passage, as judged by enhanced susceptibility via mucosal exposure and decreased survival time. Two strains of ferret CWD were described that are readily transmissible by the oral route (Perrott *et al.*, 2012). Only one strain showed accumulation of CWD prion protein (PrP^{CWD}) in lymphoid tissues and, unexpectedly, no PrP^{CWD} accumulated in the pharyngeal tonsil of inoculated ferrets. This observation led us to challenge the assumption that the tonsil is an important site for initial transduction of CWD and to investigate further the transmission and pathogenesis of CWD infection in ferrets after mucosal routes of exposure. Here, we report the broad distribution of PrP^{CWD} in ferrets inoculated with the lymphotropic strain of ferret-adapted CWD and the most likely mucosal surfaces to contribute to the acquisition and shedding of ferret-adapted CWD.

RESULTS

Summary data

The strain of ferret-adapted CWD [University of Wisconsin isolate (UWI)] used in these studies demonstrated broad prion distribution in lymphoid tissues following inoculation by the i.c. and oral *per os* (p.o.) routes (Perrott *et al.*, 2012). The p.o. and oropharyngeal (o.ph.) versus nasopharyngeal (n.ph.) routes of challenge provided the most pertinent information on the peripheral distribution of PrP^{CWD} and are thus emphasized in this report (Tables 1 and S1, available in JGV Online).

Mucosal exposures

Variations in mucosal exposure (passage 5) were studied by inoculations that targeted either the upper or lower gastrointestinal tract. Anaesthetized ferrets were positioned for safety and to maximize the effectiveness of the fluid-tight seal in the oesophagus and trachea, thus preventing exposure of the lower respiratory or digestive tracts. Ferrets inoculated via the oral (p.o.), pharyngeal (p.h.) or intragastric (i.g.) route developed CWD with attack rates of 50 % (2/4), 50 % (2/4) and 66 % (2/3), respectively (Table 1). One i.g.-inoculated ferret died for unknown reasons. In comparison with progressive CWD in passage 4 (p.o.) and passage 5 (i.c.) ferrets, mucosal exposure in passage 5 (p.o., p.h. and i.g.) resulted in more variable clinical manifestations of disease characterized by a subtle onset and periods of clinical illness that appeared to be non-progressive. However, neither χ^2 nor *t*-test statistics demonstrated significant differences in attack rate or incubation period among the inoculation groups.

Position during exposure is significant

Observations made during exposure indicated that positioning in dorsal versus sternal recumbency influenced recovery of the inoculum. Redistribution of the inoculum onto the nasal cavity mucosa occurred in dorsal recumbency (n.ph. exposure) but not in sternal recumbency (o.ph. exposure). As a consequence, ferrets in the p.o. versus p.h. exposure groups were redesignated as n.ph. versus o.ph. The attack rate was 100 % (4/4) in the n.ph. exposure group (dorsal recumbency), whereas in the o.ph. exposure group (sternal recumbency), no animals (0/4) developed CWD (significant difference at $P=0.0455$, χ^2 test) (Fig. 1b). The shorter survival time in animals exposed via the n.ph. versus the i.g. route was not statistically significant (Fig. 1b). Thus, it appeared that the regions of the pharynx most exposed to the inoculum impacted strongly on the efficacy of CWD transmission (Fig. 1).

PrP^{CWD} in the brain of infected ferrets

PrP^{CWD} was demonstrated by both immunohistochemistry (IHC) and Western blotting (WB) in the brains of all

Table 1. Passage history and mucosal transmission of CWD (UWI isolate) in ferrets

NA, Not applicable; DR, dorsal recumbency; SR, sternal recumbency.

Passage no.	Inoculation route	Attack rate*	Mean survival period in days (range)	Clinical course progression†
4	P.o.	3/3 (100 %)	474 (456–483)	Steady
5	I.c.	4/4 (100 %)	265 (256–276)	Steady
5	P.o.	2/4 (50 %)	438 (394–482)	Variable
5	I.g.	2/3 (66 %)	502 (472–532)	Variable
5	Pharyngeal	2/4 (50 %)	540 (450–630)	Variable
5	Contact	0/4 (0 %)	NA	None
5	N.ph. (DR)	4/4 (100 %)	489 (394–630)	Variable
5	O.ph. (SR)	0/4 (0 %)	NA	None

*Number of ferrets affected/number of ferrets inoculated.
†The clinical course was described as steady if a uniform onset and progressive neurological deficit was observed, and as variable if the onset was spread out and/or the clinical signs appeared non-progressive for periods of the clinical illness.
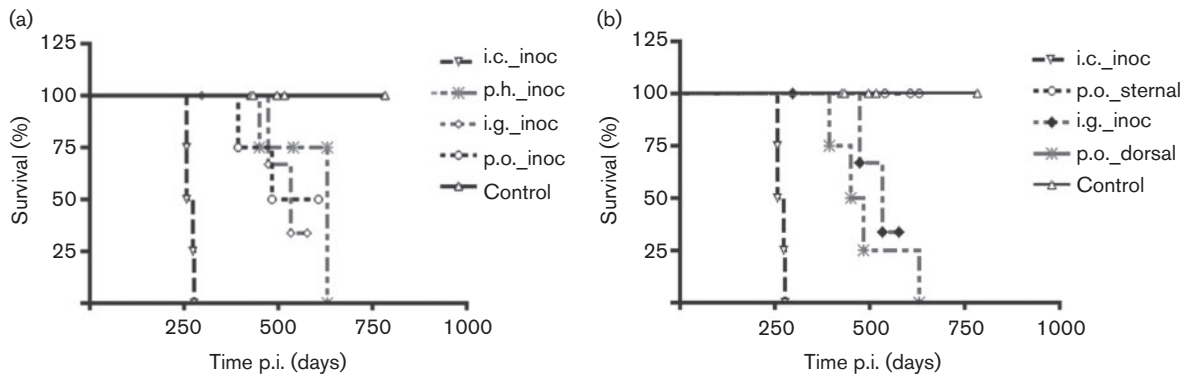
Fig. 1. Survival of ferrets following challenge by mucosal surface exposure to PrP^{CWD}. (a) Ferret survival for the p.h. (p.h._inoc), i.g. (i.g._inoc) and p.o. (p.o._inoc) routes of exposure. (b) Survival data for the same ferrets after redesignation (see Results) to n.ph. (p.o._dorsal) and o.ph. (p.o._sternal) (dorsal and sternal recumbency, respectively) exposure versus i.g. exposure (i.g._inoc). The i.c. (i.c._inoc) group was invariant. Ferret survival for the p.o._sternal and control groups overlapped.

ferrets that developed clinical signs of disease. In all groups, PrP^{CWD} occurred as coarse to punctate granular aggregates similar to those described previously (Sigurdson *et al.*, 2008). PrP^{CWD} was more prominent in the grey matter, associated with neuron support cells and the glia limitans. PrP^{CWD} was distributed symmetrically in ferrets inoculated by mucosal routes (Table S1, Fig. 2). In some situations, WB gave clearer results in the forebrain than IHC. Differences between IHC and WB scores arose when cellular prion protein (PrP^C) staining was identified by IHC. PrP^C lacks a granular, punctate appearance, and PrP^{CWD} was always assessed in the context of control slides. PrP^{CWD} could usually be discriminated by IHC, even when superimposed on the uniform background 'blush' contributed by PrP^C. IHC was especially sensitive in non-neural tissues, whereas WB seemed more sensitive in the forebrain and in some sensory structures that contained abundant PrP^C. The distribution and discrimination of PrP^{CWD} by IHC was enhanced by perfusion fixation.

The distribution of PrP^{CWD} in the brains of ferrets inoculated by the i.g. and n.ph. routes was also investigated by regional dissection and WB. PrP^{CWD} was consistently demonstrated in the brainstem, cerebellar peduncle, ventral midbrain pons, dorsal midbrain colliculus, thalamus/hypothalamus and olfactory bulb (Fig. 3). PrP^{CWD} was weakly demonstrated or absent in the hippocampus, posterior cerebral cortex, cerebral cortex, anterior olfactory cortex and lateral olfactory tract. Accumulation of PrP^{CWD} was usually greater in hindbrain than forebrain regions. Although the attack rate suggested that CWD infection resulted from non-overlapping exposure routes (p.h. vs i.g.), PrP^{CWD} distribution patterns were not sufficiently uniform within or between groups for the routes of exposure to be linked with characteristic distribution patterns (Table S1). In some animals inoculated by differing routes, however, prominent regional depositions of PrP^{CWD} were observed, such as localization in the olfactory bulb or olfactory epithelium (see

below). Ferret no. 532 (Fig. 3, Table S1) with n.ph. exposure had large amounts of PrP^{CWD} in the olfactory bulb, whereas the hindbrain versus forebrain PrP^{CWD} distribution seen in the majority of ferrets is illustrated by ferret no. 521 with i.g. exposure (Fig. 3, Table S1).

PrP^{CWD} in the upper respiratory tract and nasopharygeal mucosae

PrP^{CWD} was detected in upper respiratory tract mucosae (Fig. 4). CWD prions were prominent in the olfactory epithelium and to a lesser extent in respiratory epithelium. Punctate immunoreactivity, the hallmark of protease-resistant PrP^{CWD}, was observed in ferret no. 538, inoculated by the i.c. route (Fig. 2). Three glycoforms of ferret PrP^{CWD} have been described previously (Bartz *et al.*,1998; Sigurdson *et al.*, 2008). The predominant diglycoslyated isoform, relative molecular mass 27–30 kDa, was the most sensitive marker for PK-resistant PrP^{CWD}. The most prominent signal corresponded with the olfactory cell layer. Some evidence for a separate layer of PrP^{CWD} was seen adjacent to the basement membrane, the location of brush cells and progenitor cells. Unmyelinated nerve fibres in the lamina propria also accumulated PrP^{CWD}, as did the frontal sinus mucosa and the mucosa of the nasal turbinate (Fig. 4). Ferrets inoculated by mucosal routes showed accumulation in the central region of the olfactory epithelium, corresponding with the olfactory cell layer. Dissection and WB confirmed that PrP^{CWD} was more strongly associated with olfactory than respiratory mucosae (data not shown). Ferret no. 540 (n.ph. exposure) had relatively large amounts of PrP^{CWD} in the olfactory epithelium (Fig. 2).

PrP^{CWD} in the tongue and oropharyngeal mucosae

PrP^{CWD} was demonstrated in the dorsal mucosal surface of the tongue by dissection and WB (Fig. 5). When an area
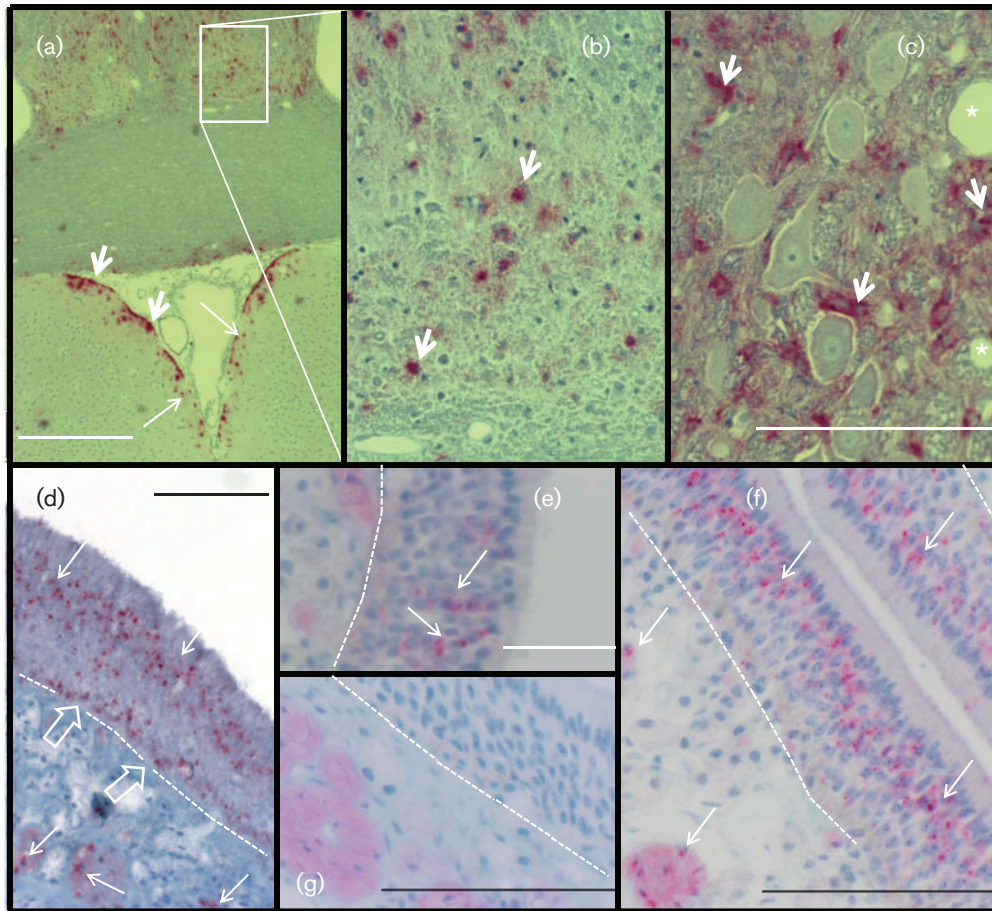
Fig. 2. PrP^{CWD} in the brain and upper respiratory tract epithelia. (a–c) IHC detection (RedMap staining) demonstrated PrP^{CWD} as punctate dots (long arrows) and coarse aggregates (short arrows) in the midbrain (a, b) and brainstem ganglion (c) of a ferret inoculated by the n.ph. route. There was symmetrical distribution of PrP^{CWD} in the thalamic region. PrP^{CWD} was associated with neurone support cells (short arrows) and a spongiform lesion (*) of brainstem ganglion. (d) PrP^{CWD} in the olfactory cell layer and in unmyelinated nerves (long arrows) beneath the olfactory epithelium of ferret no. 538 inoculated by the i.c. route. PrP^{CWD} was associated with the basal cell layer (open arrows) adjacent to the basement membrane (dotted line). (e, f) PrP^{CWD} associated with the olfactory cell layer of ferret nos 521 (e) and 540 (f), inoculated by the i.g. and n.ph. routes, respectively. (g) Olfactory epithelium of a CWD-negative ferret. Bars, 500 μm (a); 100 μm (c, f, g); 50 μm (d, e).

containing obvious taste buds was not included in the dissection, the body of the tongue, but not the pharyngeal surface, demonstrated PrP^{CWD} (Fig. 5). In the superficial layer of the tongue, PrP^{C} and PrP^{CWD} were demonstrated by IHC mainly in the taste buds (Fig. 6). PrP^{CWD} was also demonstrated sparsely in individual muscle fibres of the tongue and occasionally in nerves and small nerve ganglia (Fig. 6).

PrP^{CWD} in salivary glands and other epithelial/glandular tissues

In the parotid, mandibular and lingual salivary glands, PrP^{CWD} accumulated in the nerve cell bodies of ganglia. In the adrenal gland, small nerve ganglia and chromaffin cells in the medulla accumulated PrP^{CWD} (Fig. 6). In the pancreas, PrP^{CWD} mainly targeted small nerve ganglia and

cells in the islets of Langerhans (data not shown). Thus, PrP^{CWD} principally targeted neural and neuroendocrine rather than epithelial elements of glandular tissues.

PrP^{CWD} in lymph nodes and peripheral lymphoid tissues

PrP^{CWD} was detected in the germinal centres and lymphoid follicles of retropharyngeal, mandibular, mesenteric, colonic, duodenal, gastric and ileocecal lymph nodes. PrP^{CWD} was widespread in the lymphoid system, accumulating in the spleen and Peyer's patches (Table S1, Fig. 6). PrP^{CWD} was also associated with foci of inflammatory cells in seemingly ectopic locations such as the lachrymal gland (data not shown). Interestingly, in no animal was PrP^{CWD} demonstrated in the palatolingual tonsil, a prominent feature of the ferret caudal pharynx (Fig. 6).
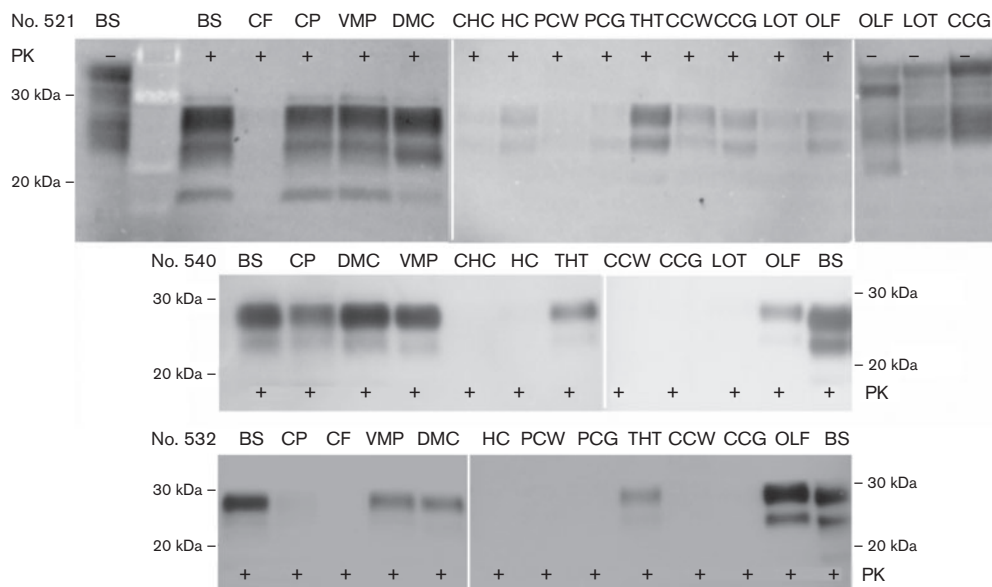
Fig. 3. Distribution of PrP^CWD in the brain of ferrets inoculated by mucosal routes. Distribution of PrP^CWD was compared in the brains of ferrets inoculated by the i.g. (no. 521) versus n.ph. (nos 540 and 532) route. Brainstem (BS), cerebellar peduncle (CP), ventral midbrain pons (VMP), dorsal midbrain colliculus (DMC), thalamus/hypothalamus (THT) and olfactory bulb (OLF) accumulated PrP^CWD. PrP^CWD was absent or less prominent in cerebellar folia (CF), caudal (CHC) and forward hippocampus (HC), posterior cortex white matter (PCW) and grey matter (PCG), cerebral cortex white matter (CCW) and grey matter (CCG) and lateral olfactory tract (LOT). Proteinase K (PK) digestion was carried out (+) or not (−), as indicated. PrP^CWD (PK+) and PrP^C (PK−) comparisons were based on 800 and 200 µg tissue equivalents (TEs), respectively.

Other sensory epithelia

PrP^CWD was demonstrated but close to the limit of detection by NaPTA-enhanced WB in the vomeronasal organ (Fig. 5), a location with implications for transmission. In contrast, PrP^CWD was abundant in the retina (data not shown).

PrP^CWD in peripheral nerves and ganglia

Although PrP^CWD was rarely demonstrated in peripheral nerves by IHC, it was detected in dorsal root ganglia associated with the pectoral (cervical cord C4–C6) and pelvic (lumbosacral cord L5–S1) limbs and in ganglia of the vagus nerve. PrP^CWD was also seen in submucosal
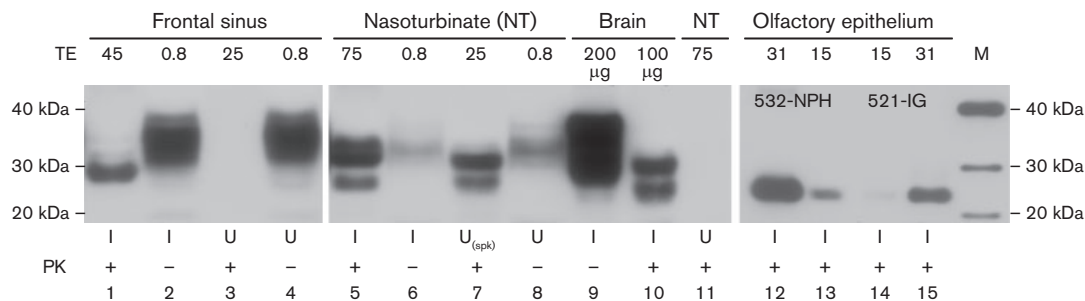

Fig. 4. PrP^CWD associated with upper respiratory tract and olfactory epithelia. PrP^C and PrP^CWD in frontal sinus, nasoturbinate (NT) and brainstem of ferrets inoculated (I) by the IC route (lanes 1–11) were compared, as shown in this composite WB. Uninoculated (U) CWD-negative ferrets showed no PrP^CWD accumulation. TEs used to demonstrate PrP^C and PrP^CWD are given in mg unless indicated otherwise. A negative sample, spiked (spk) with 200 µg TE of CWD-positive brain, showed recovery of PrP^CWD by sodium phosphotungstic acid (NaPTA) precipitation (lane 7). Dissection separation of respiratory from olfactory tissue demonstrated that PrP^CWD was more associated with olfactory tissue (data not shown). More PrP^CWD was demonstrated in the olfactory epithelium of ferret no. 532 (lanes 12 and 13) inoculated by the n.ph. route than this location in ferret no. 521 (lanes 14 and 15) inoculated by the i.g. route. M, Molecular mass markers (kDa).
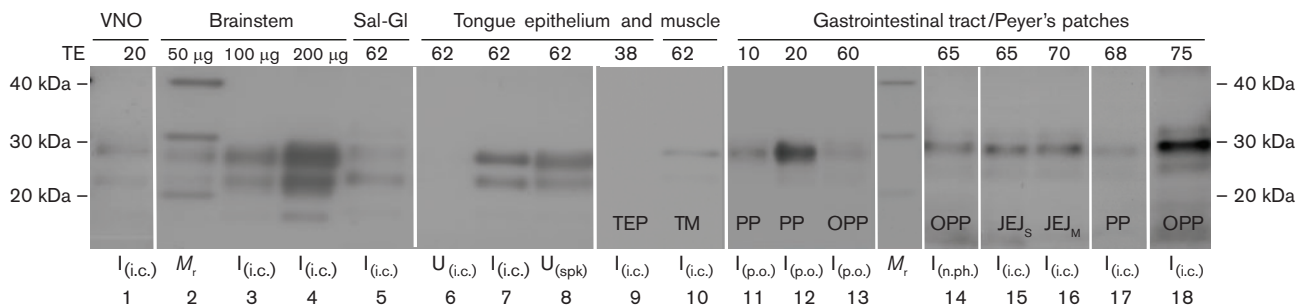
Fig. 5. PrP^{CWD} associated with the digestive tract, salivary gland and vomeronasal organ. PrP^{CWD} was detected in the vomeronasal organ (VNO, lane 1), brainstem (lanes 2–4), salivary gland (Sal-Gl, lane 5) and the tongue (lanes 7–10) of ferrets inoculated (I) by the i.c. route, as shown in this composite WB. An uninoculated (U) CWD-negative ferret showed no PrP^{CWD} accumulation (lane 6). Tongue epithelium from a negative ferret, spiked (spk) with 200 µg TE of CWD-positive brain, showed recovery of PrP^{CWD} by NaPTA precipitation (lane 8). PrP^{CWD} was detected in Peyer's patches (PP) of ferrets inoculated by the p.o. and i.c. routes (lanes 11, 12 and 17), in the gut wall opposite Peyer's patches (OPP) in ferrets inoculated by the p.o., i.c. and n.ph. routes (lanes 13, 14 and 18) and equally in the serosal (JEJ$_S$) and mesenteric (JEJ$_M$) borders of the jejunum of an i.c.-inoculated ferret (lanes 15 and 16, respectively). PrP^{CWD} was recovered from tongue muscle (TM) but not tongue epithelium (TEP) in a trial that evaluated the most superficial layer of epithelium (lanes 9 and 10). TEs are given in mg unless indicated otherwise. M_r, Molecular mass markers (kDa). The molecular mass markers were also included in lane 2 together with the brainstem sample.

ganglia and myenteric ganglia (Fig. 6) and in the vagus and sciatic nerve (data not shown).

DISCUSSION

An interesting finding of these studies was the apparent enhanced permissiveness to infection associated with exposure targeted to the nasopharyngeal mucosa. Other studies employing either nasal or aerosol prion exposure have also inferred enhanced efficacy over oral inoculation (Bessen et al., 2010; Denkers et al., 2010; Kincaid & Bartz, 2007; Kincaid et al., 2012). Nasal regions adjacent to the pharynx, exposed through anaesthetic position variation, implicated the upper respiratory tract mucosa as the site most likely to facilitate prion entry. Whilst these were clear-cut results (100 vs 0 %), relatively few ferrets were challenged in experiments not originally designed to show an effect due to positioning. Thus, there was some statistical support for differentiating nasopharyngeal entry from oropharyngeal entry but only a trend to suggest that the n.ph. route was more permissive than the lower digestive tract (100 vs 50 %), by i.g. or oral routes. The limited number of ferrets in the study and the end-point-based assessment of PrP^{CWD} distribution warrant a cautious interpretation of results.

Whilst superficial lesions in the lingual mucosa have been shown to greatly facilitate oral infection by CWD, scrapie and TME prions (Bartz et al., 2005; Carp, 1982; Denkers et al., 2011), no trauma to the mucosal surface was required for efficient CWD prion infection in ferrets exposed by the n.ph. route. However, studies by Bessen et al. (2012) have shown that damage to nasal epithelial integrity can enhance susceptibility by this route of prion exposure.

In contrast to our observations in ferrets, the tonsils of deer show early and sustained accumulation of PrP^{CWD}, affording reliable antemortem diagnosis (O'Rourke et al., 2003; Schuler et al., 2005; Sigurdson et al., 1999; Spraker et al., 2002c; Wild et al., 2002) and suggesting the tonsil as a site for initiation of infection (Sigurdson et al., 1999; Spraker et al., 2002a). Our studies suggest that adjacent anatomical locations in addition to the tonsil are likely to be permissive for prion entry in the native host. Tonsillar tissue in cervids is extensively folded and recessed in crypts, whereas the ferret tonsil is prominent and exposed in the caudal pharynx. Whilst the tonsil-associated epithelial barrier may differ between ferrets and deer, a reason for the lack of PrP^{CWD} in the ferret tonsil was not determined.

Lack of infection after o.ph. exposure was most likely a mucosal barrier effect due to stratified squamous epithelium. In contrast, permissive respiratory mucosa is pseudo-stratified, extensive and endowed with nerves and nasal-associated lymphoid tissue. IHC confirmed the presence of PrP^{CWD} in taste buds and the possibility of PrP^{CWD} shedding from this epithelial surface. Taste buds accumulate prions centrifugally following i.c. inoculation of hamsters with the HY strain of TME (DeJoia et al., 2006). Our study extends these findings to mucosal challenge and another host. Lack of disease transmission following o.ph. exposure alone indicates that taste buds were not an entry portal under our challenge conditions.

The trend for a longer incubation period following i.g. exposure may have resulted from a longer migration distance for PrP^{CWD}, prior to entry to the CNS, if trafficking of PrP^{CWD} occurred in nerves. If PrP^{CWD} was distributed by lymphatics/blood, then the trend towards
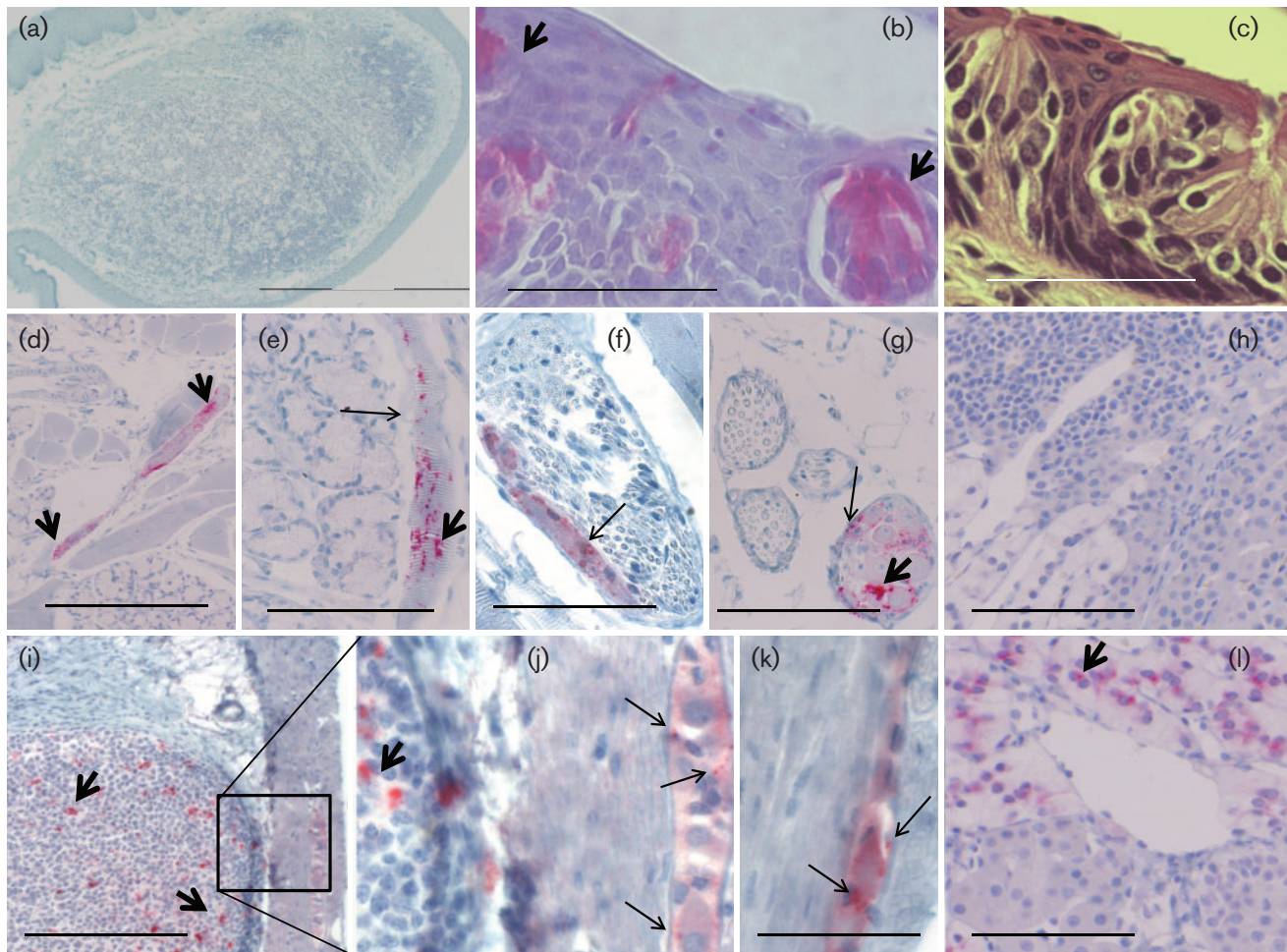
Fig. 6. Location of PrP^{CWD} in peripheral tissues. PrP^{CWD} was revealed as punctate dots (long arrows) and coarse aggregates (short arrows) by IHC (RedMap staining). (a) CWD-positive ferrets, inoculated by the n.ph. or any other route, did not accumulate PrP^{CWD} in the tonsil. (c) Haematoxylin and eosin staining of taste bud and taste pore. (b, d–g, i–l) PrP^{CWD} was seen in tastebuds (b), muscle (d, e), nerve bundles (f), ganglia (g), Peyer's patches [i, j (amplification of inset)], myenteric plexus (j, k) and adrenal medulla (l) of ferrets inoculated by the n.ph. route. (h) PrP^{CWD} was not present in uninoculated ferrets, for example the adrenal medulla of a negative ferret. Disease-specific PrP^{CWD}, with a punctate appearance, was superimposed on the PrP^C uniform 'blush' in sensory structures/nervous tissue (b, f, g, j, k). Bars, 1.0 mm (a); 100 μm (b, c, k); 400 μm (d, i); 200 μm (e–h, l).

longer incubation may have been dose related, as evidenced by the attack rate. Variable accumulation of PrP^{CWD} in Peyer's patches, mucosal ganglia and the mesenteric and root of mesentery lymph nodes of these ferrets did not enable the initiation site to be resolved.

The distribution of PrP^{CWD} in ferrets inoculated with the UWI isolate of ferret CWD (Perrott *et al.*, 2012) was similar to that in deer (Sigurdson *et al.*, 2001, 2002; Spraker *et al.*, 2002a). PrP^{CWD} was demonstrated along vagal pathways and in dorsal root ganglia, mucosal ganglia, neuroendocrine tissues and peripheral nerves, indicating that nerve transport may also be an important means of PrP^{CWD} trafficking in ferrets. PrP^{CWD} was seen in germinal centres in lymphoid tissues, ectopic mononuclear cell foci,

muscle, ganglia within exocrine and endocrine glands, and specialized sensory epithelia. CWD prions are broadly distributed in peripheral tissues, body fluids and excreta of infected cervids (Angers *et al.*, 2006, 2009; Haley *et al.*, 2009a, b; Mathiason *et al.*, 2006; Sigurdson *et al.*, 2001, 2002; Spraker *et al.*, 2002a), facilitating environmental contamination. Although we did not examine secreta or excreta, the widespread distribution of PrP^{CWD} in ferrets resembles that in deer.

Other prion transmission studies (Bessen *et al.*, 2010, 2012; Denkers *et al.*, 2010; Haybaeck *et al.*, 2011; Kincaid & Bartz, 2007; Kincaid *et al.*, 2012; Sbriccoli *et al.*, 2009) provide a basis for further examination of prion transmission via the nasal mucosa. The greater efficacy of droplet application of

TME prions to the external nares of hamsters compared with conventional oral inoculation provided some of the first evidence for prion entry by the respiratory route (Kincaid & Bartz, 2007). The earliest site of prion replication was in the nasal-associated lymphoid tissue (Kincaid & Bartz, 2007). Sbriccoli *et al.* (2009) extended these observations in hamster scrapie, ruling out direct extension from the olfactory mucosa to the brain (cranial nerve 1) but opening up the possibility of trigeminal (cranial nerve 5) involvement. The efficient transmission of prions by aerosols (Denkers *et al.*, 2010; Haybaeck *et al.*, 2011) reinforces the concept of a permissive nasal mucosal portal of prion entry. Whilst the exact channel(s) of prion entry for aerosolized versus droplet challenge may differ, the recent studies of Kincaid *et al.* (2012) demonstrated both paracellular and transcellular (M cell) mechanisms of transport across an initial entry site in the nasal mucosa of hamsters.

Ferret nos 540 and 532 (n.ph. exposure) in the current study had relatively large amounts of PrP^{CWD} in the olfactory epithelium and olfactory bulb, respectively. Although we investigated the lateral olfactory tract projection(s) described in ferrets (Dennis & Kerr, 1975) in order to show distribution patterns reflective of n.ph. versus i.g. exposure, no strong conclusions regarding the initiation of CWD infection could be drawn. It was tenable that these animals may have acquired CWD via the olfactory mucosa. It was also possible that initiation of infection was different for ferrets in the same exposure group, given that nasal cavity mucosae contain nociceptor-like cells (brush cells) that interact with the trigeminal nerve and defensive host-cell populations. The hamster model of TME implicates regional lymphocentre involvement (Kincaid & Bartz, 2007), yet the very early observations in this challenge system did not implicate nasal-associated lymphoid tissue (Kincaid *et al.*, 2012). Sbriccoli *et al.* (2009) concluded that, in hamsters, the brainstem nuclei acquire PrP^{CWD} first, and centripetal spread from the nares to the brain via the olfactory nerve does not occur (Sbriccoli *et al.*, 2009). However, as the studies of Bessen *et al.* (2010) demonstrated centrifugal excretion of prions via nasal olfactory receptor (neuroepithelial) cells, it is clear that more work is merited to understand better these intriguing aspects of prion pathogenesis.

Our results indicated that CWD prions in the ferret behave similarly to those in the hamster model of TME and support the likelihood that deer potentially shed prions from olfactory mucosae, as distribution of PrP^{CWD} to olfactory regions of the brain has been described by Spraker *et al.* (1997, 2002b). PrP^{CWD} has also been described in the nasal septum of mule deer in association with lymphoid follicles (Spraker *et al.*, 2002a). Interestingly, in sheep with scrapie, the olfactory mucosa appears to be spared (Corona *et al.*, 2009), in contrast to hamsters with sheep scrapie, which show prions in the olfactory mucosa (Sbriccoli *et al.*, 2009). With ruminant hosts seemingly differing in terms of the distribution of prions in the olfactory mucosa, it is interesting to note that ferret CWD appears to share similarities with hamster TME and hamster scrapie.

Ferret PrP^{CWD} is highly associated with mucosal surfaces, giving rise to communicable olfactory secretions and intestinal excretions. Intestinal proteases do not fully degrade PrP^{CWD}, even after prolonged incubation (M. R. Perrott, unpublished data), suggesting that environmental contamination via excreta is feasible. It would be interesting to investigate whether or not ferret CWD is transmissible by natural contact, using a trial design with a wide range of ferret-to-ferret interactions and fomite exposure. Notwithstanding uncertainty regarding lateral or environmental spread of ferret CWD, this host is a tractable species with which to investigate phenomena associated with prion transmission and CWD.

METHODS

Inocula. Brain homogenates from CWD-infected mule deer and the passages in ferrets have been described previously (Bartz *et al.*, 1998; Perrott *et al.*, 2012; Sigurdson *et al.*, 2008). The infectious deer brain material was a gift from Drs Elizabeth Williams and Michael Miller (Colorado Division of Wildlife, CO, USA). Brain tissue from ferrets was harvested aseptically and homogenized (10 and 25 %, w/v), as described previously (Perrott *et al.*, 2012).

Ferrets. Domestic ferrets (*Mustela putorius furo*) were obtained from Marshall Farms (North Rose, NY, USA) as neutered weanlings at 6–8 weeks of age. The husbandry and passage histories of the ferrets prior to passage 5 for i.c., i.p. and p.o. inoculation have been described elsewhere (Perrott *et al.*, 2012; Sigurdson *et al.*, 2008). For general anaesthesia, ferrets were pre-medicated/induced with 0.05 mg atropine (Vedco) kg^{-1} and 20 mg telazol (Fort Dodge Laboratories) kg^{-1}, intubated and maintained with isoflurane/O$_2$. Approval for the studies was obtained from the Animal Care and Use Committee, Colorado State University.

Pharyngeal exposure. This experimental strategy was designed to atraumatically expose the oral pharyngeal mucosa to CWD inoculum without exposure of the lower gastrointestinal tract. An endotracheal tube was placed and cuff inflated. A composite device – a no. 12 French feeding tube and inflatable Foley's catheter – was inserted to prevent fluid from the pharynx entering the oesophagus. Briefly, this device employed a 4–10 mm diameter ('feeding-type') catheter such that when installed the flared end blocked the oesophageal entrance. The Foley catheter was installed inside the feeding tube, with the inflatable end extending from the feeding tube. The devices were glued together and dead space was eliminated with silicon. The composite feeding tube was lubricated, fitted and the Foley catheter inflated, ensuring retention of inoculum in the oral cavity. One millilitre of 25 % (w/v) brain inoculum was placed in the caudal pharynx and redistributed over the tongue to increase exposure. After 45 min of exposure, the inoculum was recovered and the oral cavity rinsed to remove residual inoculum. Ferrets were positioned in dorsal recumbency, which allowed exposure of the nasopharygeal mucosa, or in sternal recumbency, which did not.

I.g. exposure. The lubricated cut end of a no. 12 French feeding catheter was passed as far as the distal oesophagus. A narrow paediatric catheter was introduced through the feeding catheter, into the stomach, and 1 ml of the 25 % (w/v) suspension of inoculum was injected and flushed into the stomach with saline.

Controls

Positive controls. One group of ferrets (*n*=4) for the pharyngeal exposure experiment was administered 1 ml 25 % (w/v) brain homogenate p.o. Juvenile ferrets were restrained while the suspension was administered to the caudal pharynx and until swallowing had occurred. A second group (*n*=4) was inoculated with 300 µl 10 % (w/v) brain homogenate by the i.c. route.

Negative controls. One group of ferrets (*n*=4) was administered normal (CWD-negative) ferret brain suspension by both the i.c. and p.o. route.

Experimental groups. The experimental groups were initially p.h. and i.g. (experimental) versus p.o. and i.c. (positive controls). After reassignment, the groups were n.ph., o.ph. and i.g. (experimental) versus i.c. (positive control).

Statistical analysis. Survival charts and the results of statistical analyses for reporting significance were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, http://www.graphpad.com).

WB. Tissues samples were weighed and homogenized to 10 % (w/v) in PBS (pH 7.4). Benzonase and $MgCl_2$ (Sigma) were added to 100 U ml^{-1} and 1.5 mM, respectively, and incubated for 45 min at 37 °C. *N*-Lauroyl sarcosine (4 %, w/v) in PBS was added (1 : 1) to samples and incubated for 30 min at room temperature. PK (Invitrogen) was added (50 µg ml^{-1}) and incubated for 1 h with constant agitation. Digestion was halted with Pefabloc SC (4 mM final concentration; Fluka). Brain region analysis was based on 800 µg TEs for PrP^{CWD} and 125 µg TE for PrP^C, as described previously (Perrott *et al.*, 2012). Modifications to extraction protocols for nerves and solid tissues were required (Moya *et al.*, 2004), and up to 62.5 mg TE was analysed using an extended protocol and NaPTA.

NaPTA modification. After low-speed clarification (1000 *g* for 1–2 min), PrP^{CWD} was precipitated using 0.3 % (w/v) NaPTA and centrifugation (18 000 *g* for 30 min). Pellets were resuspended in 15 µl 0.1 % (w/v) *N*-lauroyl sarcosine and 6.25 µl 4× sample buffer (Invitrogen) was added to the resuspended pellets, which were then boiled and separated by electrophoresis (12 % NuPAGE Bis-Tris polyacrylamide; Invitrogen). Proteins were transferred to PVDF membranes (Bio-Rad) and detected used standard methods (Wadsworth *et al.*, 2001).

Detection. The primary mAb BAR-224 (0.066 µg ml^{-1}; kindly supplied by Jacques Grassi, CEA, France) was detected using secondary goat anti-mouse HRP-conjugated Fab fragment (0.045 µg ml^{-1}; Jackson Laboratories) and a chemiluminescent kit (ECL plus; GE Healthcare), followed by film development and fluorescent signal capture (STORM 860; Molecular Dynamics).

Brain region analysis. Systematic dissection and semi-quantitative analysis of PrP^{CWD} from the different brain regions of infected ferrets were performed as described previously (Perrott *et al.*, 2012). Briefly, this involved harvesting a standard set of samples from precisely determined anatomical locations and preparing them for WB as described above.

IHC. Tissue samples were fixed by immersion in 10 % (v/v) neutral buffered formalin, or the ferret was perfused with 4 % (w/v) paraformaldehyde-lysine-periodate (PLP) and post-fixed in PLP for 48–72 h (Liu *et al.*, 2003; McLean & Nakane, 1974). Tissues were immersed in 88 % formic acid (1 h) and then rinsed overnight in water, equilibrated to 70 % ethanol and processed. Decalcification was by extended immersion in 10 % formic acid. Tissue sections were mounted onto positively charged glass slides, deparaffinized and rehydrated. Sections were treated using all or a subset of the methods listed below, optimized by tissue type: formic acid (88 %, v/v; Sigma) immersion for 5–15 min, hydrated autoclaving at 121 °C for 10–15 min in target antigen-retrieval solution (Dako) and proprietary CC1 and blocking solutions (Ventana Medical Systems). Automated IHC, performed on a Discovery immunostainer (Roche), used mAb BAR-224 (0.25–4.0 µg ml^{-1}), biotinylated universal secondary antibody (Roche), alkaline phosphatase–streptavidin conjugate, substrate chromagen (RedMap; Roche) and a counterstain. Positive- and negative-control tissue sections were included in each run. Several mAb variations, including omission of the primary reagent, substitution of an irrelevant primary reagent and substitution of complementary PrP antibodies, targeting different epitopes, confirmed the specificity of the staining reaction for each tissue type.

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